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(57) Abstract

This invention relates to activation of enzymes. More specifically, the invention relates to peroxidase enhancing agents. The invention also relates to methods of oxidizing a substrate with a source of hydrogen peroxide in the presence of a peroxidase enzyme and in the presence of a peroxidase enhancing agent. More specifically, the invention relates to a method of bleaching of dye in solutions, to a method of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, to a method of bleaching of lignin-containing material, in particular bleaching of pulp for paper production, to a method of treatment of waste water from pulp manufacturing, and to a method of enzymatic polymerization and/or modification of lignin or lignin containing material.

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ENHANCEMENT OF ENZYME REACTIONS

TECHNICAL FIELD

This invention relates to activation of enzymes. More specifically, the invention relates to peroxidase enhancing agents.

The invention also relates to methods of oxidizing a substrate with a source of hydrogen peroxide in the presence of a peroxidase enzyme and a peroxidase enhancing agent. More specifically, the invention relates to a method of bleaching of dye in solutions, to a method of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, to a method of bleaching of lignin-containing material, in particular bleaching of pulp for paper production, to a method of treatment of waste water from pulp manufacturing, and to a method of enzymatic polymerization and/or modification of lignin or lignin containing material.

BACKGROUND ART

Peroxidases (E.C. 1.11.1.7) are enzymes that catalyse the oxidation of a substrate (an electron or hydrogen donor) with hydrogen peroxide. Such enzymes are known from microbial, plant and animal origins, e.g. peroxidase from Coprinus cinereus (cf. e.g. EP 179,486). They are typically hemoproteins, i.e. they contain a heme as a prosthetic group.

Use of peroxidase together with hydrogen peroxide or a hydrogen peroxide precursor has been suggested e.g. in bleaching of pulp for paper production, in treatment of waste water from pulp production, for improved bleaching in laundry detergents, for dye transfer inhibition during laundering, and for lignin modification, e.g. in particle board production.

The compound 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate), ABTS, supplied by Boehringer Mannheim, is a chromogenic substrate, and a common peroxidase and phenol

peroxidase assay agent. These enzymes catalyse the oxidation of ABTS by hydrogen peroxide and dioxygen, respectively, producing a greenish-blue colour, which process may be monitored photometrically.

ABTS has been found to form a stable radical cation when oxidized by a laccase enzyme (polyphenol oxidase, EC 1.10.3.2), and has been proposed to act as a redox mediator for oxidation of non-phenolic lignin model compounds [Bourbonnais R, Paice M G; FEBS Lett (1990) **267** 99-102].

Studies on demethylation and delignification of kraft pulp by a laccase enzyme in the presence of ABTS showed that the extent of partial demethylation by laccase was increased in the presence of ABTS [Bourbonnais, R. and Paice, M.G; Appl. Microbiol. Biotechnol. (1992) **36** 823-827].

Certain oxidizable substrates e.g. metal ions and phenolic compounds such as 7-hydroxycoumarin (7HCm), vanillin (VAN), and p-hydroxybenzenesulfonate (pHBS), have been described as accelerators or enhancers, able to enhance bleaching reactions (cf. e.g. WO 92/18683, WO 92/18687, and Kato M and Shimizu S, Plant Cell Physiol. 1985 **26** (7), pp. 1291-1301 (cf. Table 1 in particular), or Saunders B C, et al., Peroxidase, London, 1964, p. 141 ff).

SUMMARY OF THE INVENTION

It is an object of the invention to provide an agent for enhancing the activity of peroxidase enzymes, and to provide a method of enhancing the activity of peroxidase enzymes. It has now surprisingly been found that the activity of peroxidases increases significantly in the presence of an azino compound as described herein.

Accordingly, in its first aspect, the present invention provides an agent for enhancing the activity of a peroxidase enzyme, the agent being described by the general formula

A=N-N=B

in which formula A and B, which may be identical or different, independently represents any of the substituents presented in Fig. 1;

in which substituents the symbols X and Y, which may be identical or different, independently represents carbon, nitrogen, which nitrogen may be unsubstituted or substituted with a substituent group R^5, sulfur, oxygen, selenium or tellurium;

and in which substituents the substituent groups R^1, R^2, R^3, and R^4, which may be identical or different, independently represents hydrogen, halogen, a hydroxy group, a C_1-C_3 alkoxy group, a formyl group, a carboxy group, a sulfo group, a nitro group, a C_1-C_5 alkyl group, which alkyl group may furthermore be saturated or unsaturated, or an amino group, which amino group may furthermore be unsubstituted or substituted once or twice with a substituent group R^5;

which substituent group R^5 represents halogen, a hydroxy group, a C_1-C_3 alkoxy group, a C_1-C_5 alkyl group, or an amino group.

In its second aspect, the invention provides a method of oxidizing a substrate with a peroxidase enzyme, in the presence of a source of hydrogen peroxide, and in the presence of a peroxidase enhancing agent of the invention.

In a specific aspect, the invention provides a method of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the method comprising treatment of the wash liquor with a peroxidase enzyme in the presence of a source of hydrogen peroxide and in presence of a peroxidase enhancing agent of the invention.

In a particular aspect, the invention provides a detergent additive capable of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the detergent additive comprising an enzyme exhibiting peroxidase activity, a source of hydrogen peroxide and a peroxidase enhancing agent of the invention.

In another particular aspect, the invention provides a detergent composition capable of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the detergent composition comprising an enzyme exhibiting peroxidase activity, a source of hydrogen peroxide, and a peroxidase enhancing agent of the invention.

In another aspect, the invention provides a method of bleaching of lignin-containing material, in particular bleaching of pulp for paper production, the method comprising treatment of the lignin or lignin containing material with a peroxidase enzyme in the presence of a source of hydrogen peroxide and in the presence of a peroxidase enhancing agent of the invention.

In a further aspect, the invention provides a method of enzymatic polymerization and/or modification of lignin or lignin containing material, the method comprising treatment of the lignin or lignin containing material with a peroxidase enzyme in the presence of a source of hydrogen peroxide and in presence of a peroxidase enhancing agent of the invention.

In a yet further aspect, the invention provides a method of treatment of waste water, e.g. waste water from the chemical or pharmaceutical industry, the method comprising treatment of the waste water with a peroxidase enzyme in the presence of a source of hydrogen peroxide and in the presence of a peroxidase enhancing agent of the invention.

BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated by reference to the accompanying drawings, in which:

Fig. 1 shows the substituents II, III, IV, and V, of the general formula I according to the invention;

Fig. 2 shows a comparison of a peroxidase enhancing agent of the invention (ABTS) and pHBS, applied to bleaching of Methyl Orange by a Coprinus cinereus peroxidase (1: pHBS, 20 μM

H_2O_2; 2: pHBS, 200 µM H_2O_2; 3: ABTS, 20 µM H_2O_2; 4: ABTS, 200 µM H_2O_2);

Fig. 3 shows accelerated bleaching of Methyl Orange by a Coprinus cinereus peroxidase in the presence of varying concentrations of a peroxidase enhancing agent of the invention (ABTS) (1: 0 µM ABTS; 2: 1 µM ABTS; 3: 5 µM ABTS; and 4: 10 µM ABTS);

Fig. 4 shows a comparison of the initial bleaching rates during bleaching of Direct Blue 1 (DB1) at pH 10.5 (□ ABTS, 1 nM peroxidase; ♦ VAN, 100 nM peroxidase; ■ 7HCm, 100 nM peroxidase; ▲ pHBS, 100 nM peroxidase); and

Fig. 5 shows a comparison of the initial bleaching rates during bleaching of DB1 at pH 8.8 (and pH 10.5) (□ ABTS pH 8.8; ♦ VAN pH 8.8; ■ 7HCm pH 8.8; ◊ ABTS pH 10.5; and ▲ pHBS pH 8.8).

DETAILED DISCLOSURE OF THE INVENTION

The Peroxidase Enhancing Agent

The present invention relates to the use of a known chemical compound for enhancing the activity of peroxidase enzymes. Accordingly, the invention provides an agent capable of enhancing the effect of a peroxidase enzyme.

The agent is an azino compound described by the general formula I:

A=N-N=B

in which formula the symbols A and B, which may be identical or different, independently represents any of the substituents II, III, IV, and V, presented in Fig. 1;

in which substituents the symbols X and Y, which may be identical or different, independently represents carbon, nitrogen, which nitrogen may be unsubstituted or substituted with a substituent group R^5, sulfur, oxygen, selenium or tellurium;

and in which substituents the substituent groups R^1, R^2, R^3, and R^4, which may be identical or different, independently represents hydrogen, halogen, a hydroxy group, a C_1-C_3 alkoxy group, a formyl group, a carboxy group, a sulfo group, a nitro group, a C_1-C_5 alkyl group, which alkyl group may furthermore be saturated or unsaturated, linear or branched, or an amino group, which amino group may furthermore be unsubstituted or substituted once or twice with a substituent group R^5;

which substituent group R^5 represents halogen, a hydroxy group, a C_1-C_3 alkoxy group, a C_1-C_5 alkyl group, or an amino group.

The peroxidase enhancing agent of the invention may be in free form or in the form of an addition salt.

In preferred embodiments, the substituent groups R^1, R^2, R^3, and R^4, which may be identical or different, independently represents hydrogen, halogen, a hydroxy group, a C_1-C_3 alkyl group, or a sulfo group. Preferably, the halogen is fluoro, chloro, or bromo. Preferably, the C_1-C_3 alkyl group is methyl, ethyl, propyl, or isopropyl.

In preferred embodiments, the substituent group R^5 represents halogen, a hydroxy group, a C_1-C_3 alkoxy group, a C_1-C_3 alkyl group, or an amino group.

In a most preferred embodiment, a peroxidase enhancing agent of the invention is 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonate). This compound, abbreviated ABTS, is a chromogenic substrate, and a common peroxidase and phenol oxidase assay agent.

It has, moreover, been demonstrated that ABTS, contrary to the enhancers known and described above, is capable of acting as a peroxidase enhancing agent at highly alkaline conditions, i.e. above pH 9. This feature allows ABTS to be implemented into e.g. detergent compositions, intended for performance in the range pH 7-13, particularly the range pH 8-12, preferably the range pH 9-11.

Methods of Oxidizing in the Presence of Peroxidases

In another aspect, the invention provides a method of oxidizing a substrate with a source of hydrogen peroxide in the presence of a peroxidase enzyme, the method being characterized by the presence of a peroxidase enhancing agent of the general formula

A=N-N=B

in which formula the symbols A and B, which may be identical or different, independently represents any of the substituents II, III, IV, and V, presented in Fig. 1;

in which substituents the symbols X and Y, which may be identical or different, independently represents carbon, nitrogen, which nitrogen may be unsubstituted or substituted with a substituent group R^5, sulfur, oxygen, selenium or tellurium;

and in which substituents the substituent groups R^1, R^2, R^3, and R^4, which may be identical or different, independently represents hydrogen, halogen, a hydroxy group, a C_1-C_3 alkoxy group, a formyl group, a carboxy group, a sulfo group, a nitro group, a C_1-C_5 alkyl group, which alkyl group may furthermore be saturated or unsaturated, or an amino group, which amino group may furthermore be unsubstituted or substituted once or twice with a substituent group R^5;

which substituent group R^5 represents halogen, a hydroxy group, a C_1-C_3 alkoxy group, a C_1-C_5 alkyl group, or an amino group.

The peroxidase enhancing agent of the invention may be in free form or in the form of an addition salt.

In preferred embodiments, the substituent groups R^1, R^2, R^3, and R^4, which may be identical or different, independently represents hydrogen, halogen, a hydroxy group, a C_1-C_3 alkyl group, or a sulfo group. Preferably, the halogen is fluoro, chloro, or bromo. Preferably, the C_1-C_3 alkyl group is methyl, ethyl, propyl, or isopropyl.

In preferred embodiments, the substituent group R^5 represents halogen, a hydroxy group, a C_1-C_3 alkoxy group, a C_1-C_3 alkyl group, or an amino group.

In a further preferred embodiment, the peroxidase enhancing agent of the invention is 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonate) (ABTS).

The enzyme employed in the method of the invention may be any peroxidase enzyme comprised by the enzyme classification EC 1.11.1.7. Such enzymes are known from microbial, plant and animal origins.

Preferably, the peroxidase employed in the method of the invention is producible by plants (e.g. horseradish peroxidase) or microorganisms such as fungi or bacteria. In a further preferred embodiment, the peroxidase is derived from Coprinus, e.g. C. cinereus or C. macrorhizus, or from Bacillus, e.g. B. pumilus, particularly a peroxidase according to PCT/DK 90/00260.

The peroxidase may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said peroxidase as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the peroxidase, in a culture medium under conditions permitting the expression of the peroxidase and recovering the peroxidase from the culture.

Particularly, a recombinantly produced peroxidase is a peroxidase derived from a Coprinus sp., in particular Coprinus macrorhizus or cinereus according to WO 92/16634.

The peroxidase enhancing agent of the invention may be present in concentrations of from 0.01 to 100 μM, more preferred 0.1 to 50 μM, most preferred 1 to 10 μM.

The source of hydrogen peroxide may be hydrogen peroxide or a hydrogen peroxide precursor, e.g. percarbonate or perborate, or a hydrogen peroxide generating enzyme system, e.g. an oxidase and a substrate for the oxidase. Hydrogen peroxide may be added at the beginning or during the process, e.g. in an amount of 0.001-5 mM, particularly 0.01-1 mM. When using

Coprinus peroxidase, 0.01-0.25 mM H_2O_2 is preferred, and with B. pumilus peroxidase 0.1-1 mM H_2O_2.

Industrial Applications

Methods according to the invention of oxidizing a substrate with a source of hydrogen peroxide in the presence of a peroxidase enzyme find various industrial applications.

In a preferred embodiment, the method of the invention finds application for bleaching of dye in solutions.

In another embodiment, the method of the invention finds application for dye transfer inhibition, e.g. for treatment of dyed textiles (cf. e.g. WO 92/18687) or during laundering (cf. e.g. WO 91/05839).

Accordingly, in a specific embodiment, the invention provides a method for inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the method comprising treatment of the wash liquor with a peroxidase enzyme in the presence of a source of hydrogen peroxide, and the presence of a peroxidase enhancing agent of the invention. The textile dye may be a synthetic dye such as an azo dye, or a natural or nature-identical dye.

In a third embodiment, the method of the invention finds application in bleaching of pulp for paper production. The use of a peroxidase together with hydrogen peroxide or a hydrogen peroxide precursor in bleaching of paper pulp has been described in e.g. SE 88/0673 and US 4,690,895.

Accordingly, the invention provides a method for bleaching of lignin-containing material, in particular bleaching of pulp for paper production, which method comprises treatment of the lignin or lignin containing material with a peroxidase enzyme in the presence of a source of hydrogen peroxide and in the presence of a peroxidase enhancing agent of the invention.

In a fourth embodiment, the method of the invention finds application for lignin modification, e.g. in particle board production. Binders for producing wood composites such as

fibre boards and particle boards can be made from peroxidase treated lignin (cf. US 4,432,921).

Accordingly, the invention provides a method for enzymatic polymerization and/or modification of lignin or lignin containing material, which method comprises treatment of the lignin or lignin containing material with a peroxidase enzyme in the presence of a source of hydrogen peroxide, and the presence of a peroxidase enhancing agent of the invention.

In a fifth embodiment, the method of the invention finds application in treatment of waste water e.g. waste water from the chemical or pharmaceutical industry, from dye manufacturing, from the textile industry, or from pulp production (cf. e.g. US 4,623,465, or JP-A 2-31887).

In a more specific aspect, the invention provides a method for treatment of waste water from dye manufacturing, from textile industry, or from pulp manufacturing, the method comprising treatment of the waste water with a peroxidase enzyme in the presence of a source of hydrogen peroxide and in the presence of a peroxidase enhancing agent of the invention.

Detergent Compositions

According to the invention, the peroxidase enhancing agent may be added as a component of a detergent composition.

In a specific aspect, the invention provides a detergent additive capable of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the detergent additive comprising an enzyme exhibiting peroxidase activity, a source of hydrogen peroxide, and a peroxidase enhancing agent of the invention. The detergent additive may additionally comprise one or more other enzymes conventionally used in detergents, such as proteases, lipases, amylases, or cellulases.

Preferably, the detergent additive is provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or in a protected form.

In another specific aspect, the invention provides a detergent composition capable of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the detergent composition comprising an enzyme exhibiting peroxidase activity, a source of hydrogen peroxide and a peroxidase enhancing agent of the invention.

The peroxidase enhancing agent of the invention may be included in the detergent as a part of a peroxidase system, comprising a peroxidase enzyme, a source of hydrogen peroxide, and the peroxidase enhancing agent of the invention.

The peroxidase system may be included in the detergent composition in the form of a non-dusting granulate, a liquid, in particular a stabilized liquid, or in a protected form. Non-dusting granulates may be produced, e.g. as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. A protected form of the peroxidase system may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules or liquid. A liquid detergent may be aqueous, typically containing up to 90% water and 0-20% organic solvent.

The detergent composition comprises surfactants which may be anionic, nonionic, cationic, amphoteric or a mixture of these types. The detergent will usually contain 0-50% anionic surfactant such as linear alkylbenzenesulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (AS), alcohol ether sulfate (AES) or soap. It may also contain 0-40% nonionic surfactant such as nonyl phenol ethoxylate or alcohol ethoxylate. Furthermore, it may contain a polyhydroxy fatty acid amide surfactant (e.g. as described in WO 92/06154).

The pH (measured in aqueous detergent solution) will usually be neutral or alkaline, e.g. 7-11.

The detergent may contain 1-40% of a detergent builder such as zeolite, phosphate, phosphonate, citrate, NTA, EDTA or DTPA, alkenyl succinic anhydride, or silicate, or it may be unbuilt (i.e. essentially free of a detergent builder). It may also contain other conventional detergent ingredients, e.g. fabric conditioners, foam boosters, bleaching agents, e.g. perborate, percarbonate, tetraacetyl ethylene diamine (TAED), or nonanoyloxybenzenesulfonate (NOBS), anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil re-deposition agents, stabilizing agents for enzymes, foam depressors, dyes, bactericides, optical brighteners or perfumes.

The detergent composition may additionally comprise one or more other enzymes conventionally used in detergents such as proteases, lipases, amylases, and cellulases.

Particular forms of detergent composition within the scope of the invention include:

a) A detergent composition formulated as a detergent powder containing phosphate builder, anionic surfactant, nonionic surfactant, silicate, alkali to adjust to desired pH in use, and neutral inorganic salt.

b) A detergent composition formulated as a detergent powder containing zeolite builder, anionic surfactant, nonionic surfactant, acrylic or equivalent polymer, silicate, alkali to adjust to desired pH in use, and neutral inorganic salt.

c) A detergent composition formulated as an aqueous detergent liquid comprising anionic surfactant, nonionic surfactant, humectant, organic acid, alkali, with a pH in use adjusted to a value between 7 and 10.5.

d) A detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant consisting essentially of linear alkoxylated primary alcohol, phosphate builder, alkali, with a pH in use adjusted to a value between about 7 and 10.5.

e) A detergent composition formulated as a detergent powder in the form of a granulate having a bulk density of at least 600 g/l, containing anionic surfactant and nonionic surfactant, phosphate builder, sodium silicate, and little or substantially no neutral inorganic salt.

f) A detergent composition formulated as a detergent powder in the form of a granulate having a bulk density of at least 600 g/l, containing anionic surfactant and nonionic surfactant, zeolite builder, sodium silicate, and little or substantially no neutral inorganic salt.

g) A detergent composition formulated as a detergent powder containing anionic surfactant, nonionic surfactant, acrylic polymer, fatty acid soap, sodium carbonate, sodium sulfate, clay particles, and sodium silicate.

h) A liquid compact detergent comprising 5-65% by weight of surfactant, 0-50% by weight of builder and 0-30% by weight of electrolyte.

The following examples further illustrate the present invention, and they are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

Bleaching of Methyl Orange

Accelerated bleaching of Methyl Orange (Merck) catalysed by a recombinantly produced Coprinus cinereus peroxidase (rCiP), obtained according to WO 92/16634, and hydrogen peroxide in the presence of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS, supplied by Boehringer Mannheim) or para-hydroxybenzene sulfonate (pHBS, supplied by Sigma) is shown in Fig. 2. The following conditions were used:

10 nM rCiP
25 μM Methyl Orange
50 μM ABTS or para-hydroxybenzene sulfonate
20 and 200 μM hydrogen peroxide

50 mM Britton & Robinson buffer, pH 8.8
30°C thermostat

Reagents were mixed in a 1 cm cuvette, and the bleaching was started by addition of hydrogen peroxide. The bleaching was detected spectrophotometrically at 465 nm, which is the absorption peak of this dye. Bleaching was followed with respect to time over a span of 10 min.

EXAMPLE 2

Bleaching of Methyl Orange

Accelerated bleaching of Methyl Orange (Merck) catalysed by a recombinantly produced Coprinus cinereus peroxidase (rCiP), obtained according to WO 92/16634, and hydrogen peroxide in the presence of varying concentrations of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS, supplied by Boehringer Mannheim) is shown in Fig. 3. The following conditions were used:

10 nM rCiP
25 μM Methyl Orange
0, 1, 5 and 10 μM ABTS
200 μM hydrogen peroxide
50 mM Britton & Robinson buffer, pH 8.8
30°C thermostat

Mixture, start and detection of the bleaching are as described in Example 1.

EXAMPLE 3

Bleaching of Direct Blue 1

The initial bleaching of Direct Blue 1 (DB1) by recombinantly produced Coprinus cinereus peroxidase (rCiP), obtained according to WO 92/16634, using ABTS as accelerator, was compared to the best of the hitherto known accelerators: 7-

hydroxycoumarin (7HCm), vanillin (VAN), and p-hydroxybenzene sulfonate (pHBS). The following conditions were used:

1 nM rCiP or 100 nM rCiP (at pH 10.5)
0, 10, 25, 50, or 75 μM accelerator, respectively
50 mM Britton & Robinson buffer, pH 8.8 or 10.5, respectively
20 μM hydrogen peroxide

Reagents were mixed in a 1 cm cuvette, and the bleaching was started by addition of hydrogen peroxide. The bleaching was detected spectrophotometrically at 610 nm, which is the absorption peak of this dye. Bleaching was followed for 10 minutes, and the bleaching rates (-ΔmAbs/min) were determined from the initial (linear) reduction in absorbance.

At pH 10.5 the bleaching using 100 nm rCiP and ABTS as accelerator was so fast that bleaching was already completed before the cuvette could be placed in the spectrophotometer, the reason why the dosage of rCiP at pH 10.5 was reduced to 1 nM when used in combination with ABTS, although a dosage near 100 nM rCiP was necessary for all other (hitherto known) accelerators in order to see a significant reduction in absorbance.

The results of initial bleaching rate per minute have been illustrated in Figs. 4 and 5 as function of accelerator concentration.

EXAMPLE 4

Enhanced Dye Transfer Inhibition by ABTS

A washing trial was carried out in a Terg-o-tometer to investigate the effect of ABTS on peroxidase based dye transfer inhibition. For a comparison, also the established enhancer pHBS was tested.

Clean white tracer test pieces (cotton, Style#400 from Testfabrics, Inc., USA; bleached, but unbrightened) were washed together with nylon test pieces dyed with the azo dye Acid Red

151 (C.I. 26900; available, e.g. from Aldrich Chemical Co.). Reference test pieces were cut out of the same cotton cloth and washed in the absence of dyed fabric. The dye transfer in a given Terg-o-tometer pot was measured as the Hunter color difference

$$\Delta E = \sqrt{(\Delta L)^2+(\Delta a)^2+(\Delta b)^2}$$

between the tracer pieces in that pot and the above reference pieces, the Hunter L, a, and b values being evaluated from remission data obtained with an unfiltered daylight source on a Datacolor Elrephometer 2000.

The detergent solution for the washing trial was made up using 4.5 g/l of a commercially available European high-pH powder detergent containing no bleach and no optical brightener. The water used was tap water mixed with demineralized water in the ratio 1:2; the mixture had a hardness equivalent to approx. 1.1 mM Ca^{2+}.

The detailed experimental conditions were:

Duration of wash:	15 min.
Terg-o-tometer agitation:	70 rotations/min.
Temperature:	35°C
pH:	Adjusted to 10.5 with NaOH prior to addition of peroxidase system
Textile load:	Approx. 6 g nylon dyed with acid Red 151 and 1 g white cotton per litre washing liquor
Peroxide source:	In all cases, 50 μM H_2O_2 was present together with the peroxidase
Peroxidase:	Recombinantly produced Coprinus cinereus peroxidase, obtained according to WO 92/16634, at 5 nM

After washing, the test pieces were rinsed thoroughly in cold tap water and dried in the dark overnight, after which the remission measurements were performed.

Treatments with various concentrations of the two enhancers yielded the following results:

	Hunter ΔE with respect to white, washed fabric
1μM ABTS	34.9
5μM ABTS	32.3
20μM ABTS	23.7
1μM pHBS	34.8
5μM pHBS	34.5
20μM pHBS	30.8

Differences of ≥2 units of Hunter ΔE were statistically significant.

In both cases, the peroxidase system with 1 μM enhancer provided no significant dye transfer inhibition (reference without peroxidase system not included here). However, as is seen, the ABTS system has an effect already at 5 μM of enhancer, whereas the pHBS system does not; and at 20 μM enhancer, the ABTS system has a much larger effect than the pHBS system.

CLAIMS

1. An agent for enhancing the activity of a peroxidase enzyme, **characterized** by the general formula I

A=N-N=B

in which formula the symbols A and B, which may be identical or different, independently represents any of the substituents II, III, IV, and V, presented in Fig. 1;

in which substituents the symbols X and Y, which may be identical or different, independently represents carbon, nitrogen, which nitrogen may be unsubstituted or substituted with a substituent group R^5, sulfur, oxygen, selenium or tellurium;

and in which substituents the substituent groups R^1, R^2, R^3, and R^4, which may be identical or different, independently represents hydrogen, halogen, a hydroxy group, a C_1-C_3 alkoxy group, a formyl group, a carboxy group, a sulfo group, a nitro group, a C_1-C_5 alkyl group, which alkyl group may furthermore be saturated or unsaturated, or an amino group, which amino group may furthermore be unsubstituted or substituted once or twice with a substituent group R^5;

which substituent group R^5 represents halogen, a hydroxy group, a C_1-C_3 alkoxy group, a C_1-C_5 alkyl group, or an amino group.

2. An agent according to claim 1, being 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate).

3. A method of oxidizing a substrate with a peroxidase enzyme in the presence of a source of hydrogen peroxide, **characterized** by the presence of a peroxidase enhancing agent of the general formula

A=N-N=B

in which formulae A and B, which may be identical or different, independently represents any of the substituents II, III, IV, and V, presented in Fig. 1;

in which substituents the symbols X and Y, which may be identical or different, independently represents carbon, nitrogen, which nitrogen may be unsubstituted or substituted with a substituent group R^5, sulfur, oxygen, selenium or tellurium;

and in which substituents the substituent groups R^1, R^2, R^3, and R^4, which may be identical or different, independently represents hydrogen, halogen, a hydroxy group, a C_1-C_3 alkoxy group, a formyl group, a carboxy group, a sulfo group, a nitro group, a C_1-C_5 alkyl group, which alkyl group may furthermore be saturated or unsaturated, or an amino group, which amino group may furthermore be unsubstituted or substituted once or twice with a substituent group R^5;

which substituent group R^5 represents halogen, a hydroxy group, a C_1-C_3 alkoxy group, a C_1-C_5 alkyl group, or an amino group.

4. A method according to claim 3, in which the peroxidase enhancing agent is 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate).

5. A method according to either of claims 3-4, in which the peroxidase enhancing agent is added at the beginning of, or during the process.

6. A method according to any of claims 3-5, in which the amount of peroxidase enhancing agent is in the range of from 0.01-100 μM, more preferred 0.1-50 μM, most preferred 1-10μM.

7. A method according to any of claims 3-6, in which the source of hydrogen peroxide is hydrogen peroxide or a hydrogen peroxide precursor, e.g. percarbonate or perborate, or a hydrogen peroxide generating enzyme system, e.g. an oxidase and its substrate.

8. A method according to any of claims 3-7, in which the peroxidase enzyme is horseradish peroxidase or a peroxidase enzyme derived from Coprinus, e.g. C. cinereus or C. macrorhizus, or from Bacillus, e.g. B. pumilus.

9. A method according to any of claims 3-8, applied to bleaching of dye in solutions.

10. A method according to any of claims 3-8, applied to inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the method comprising treatment of the wash liquor with a peroxidase enzyme in the presence of a source of hydrogen peroxide, **characterized** by the presence of a peroxidase enhancing agent of either of claims 1-2.

11. A detergent additive capable of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the detergent additive comprising an enzyme exhibiting peroxidase activity, a source of hydrogen peroxide, and a peroxidase enhancing agent according to either of claims 1-2.

12. A detergent additive according to claim 11, the peroxidase enhancing agent being 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate).

13. A detergent additive according to either of claims 11-12, provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or a protected enzyme.

14. A detergent composition capable of inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the detergent composition comprising an enzyme exhibiting peroxid-

ase activity, a source of hydrogen peroxide, and a peroxidase enhancing agent according to either of claims 1-2.

15. A detergent composition according to claim 14, the peroxidase enhancing agent being 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate).

16. A detergent composition according to claims 14-15, which further comprises one or more other enzymes, in particular a protease, a lipase, an amylase, a cellulase, and/or oxidases, or a mixture hereof.

17. A method according to any of claims 3-8, applied to bleaching of lignin-containing material, in particular bleaching of pulp for paper production, the method comprising treatment of the lignin or lignin containing material with a peroxidase enzyme in the presence of a source of hydrogen peroxide, **characterized** by the presence of a peroxidase enhancing agent of either of claims 1-2.

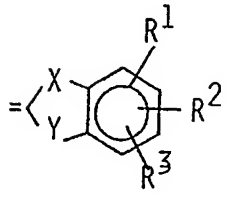
18. A method according to any of claims 3-8, applied to enzymatic polymerization and/or modification of lignin or lignin containing material, the method comprising treatment of the lignin or lignin containing material with a peroxidase enzyme in the presence of a source of hydrogen peroxide, **characterized** by the presence of a peroxidase enhancing agent of either of claims 1-2.

19. A method according to any of claims 3-8, applied to treatment of waste water, e.g. waste water from the chemical or pharmaceutical industry, the method comprising treatment of the waste water with a peroxidase enzyme in the presence of a source of hydrogen peroxide, **characterized** by the presence of a peroxidase enhancing agent of either of claims 1-2.

20. A method according to claim 19, for treatment of waste water from dye manufacturing, from textile industry, or

from pulp manufacturing, the method comprising treatment of the waste water with a peroxidase enzyme in the presence of a source of hydrogen peroxide, **characterized** by the presence of a peroxidase enhancing agent of either of claims 1-2.

(II)



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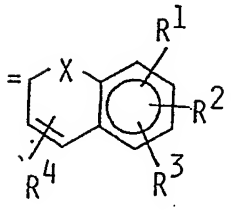

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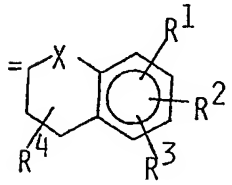

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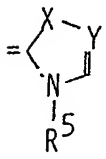


Fig. 1

RECTIFIED SHEET (RULE 91)

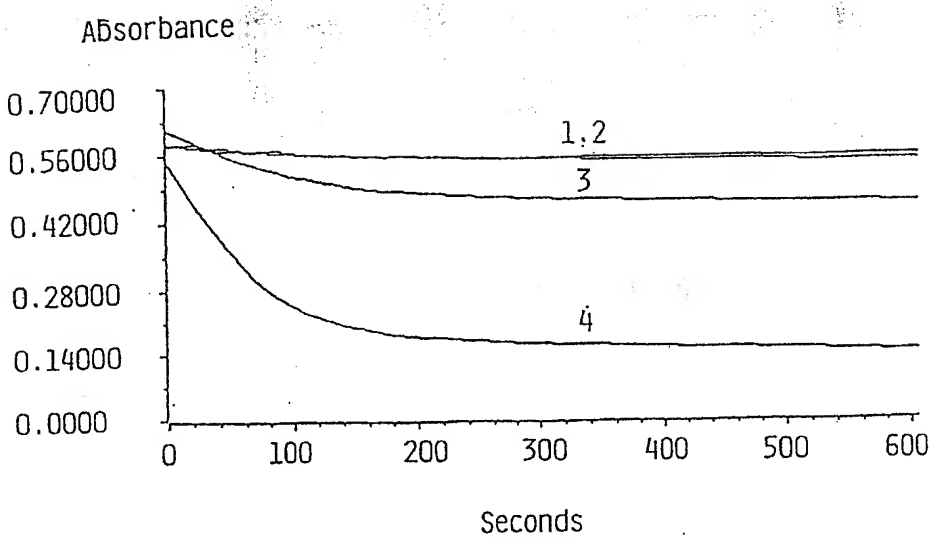
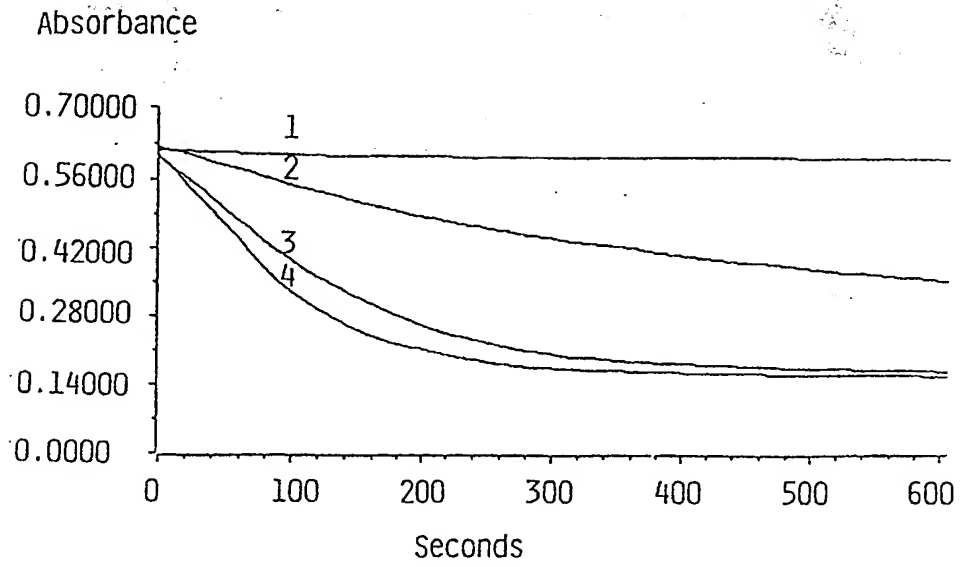


Fig. 2

RECTIFIED SHEET (RULE 91)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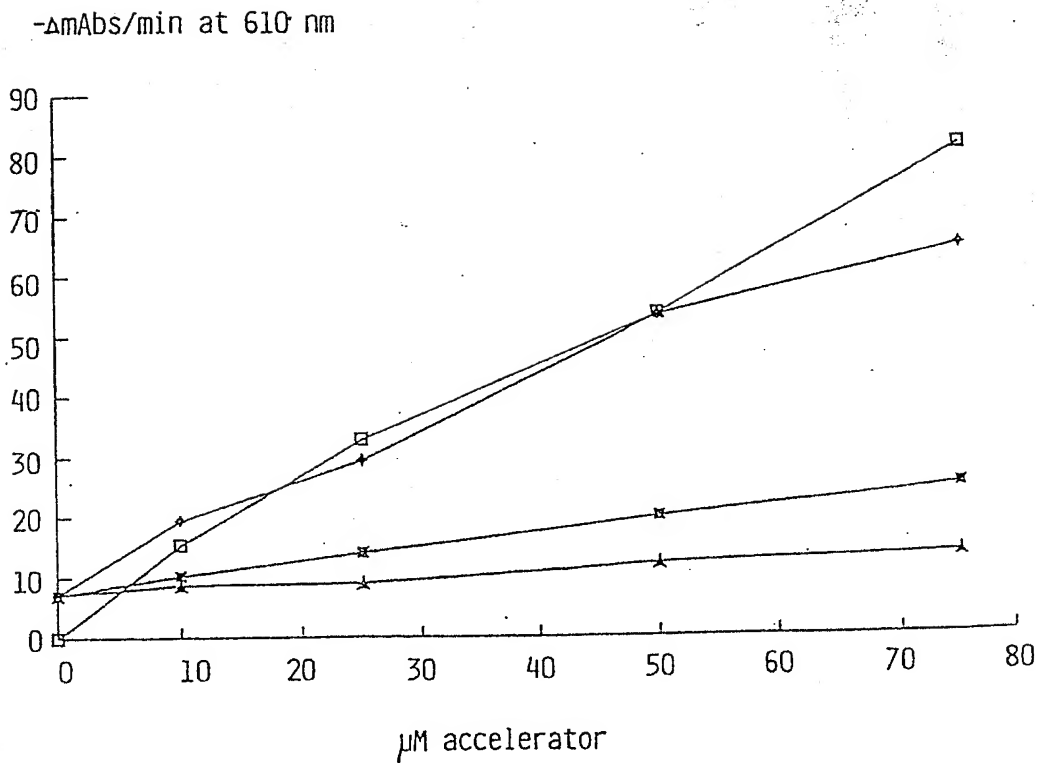
0.56000
0.42000
0.28000
0.14000
0.0000
1
2
3
4
0
100
200
300
400
500
600
Seconds

Fig. 3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90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
μM accelerator

Fig. 4

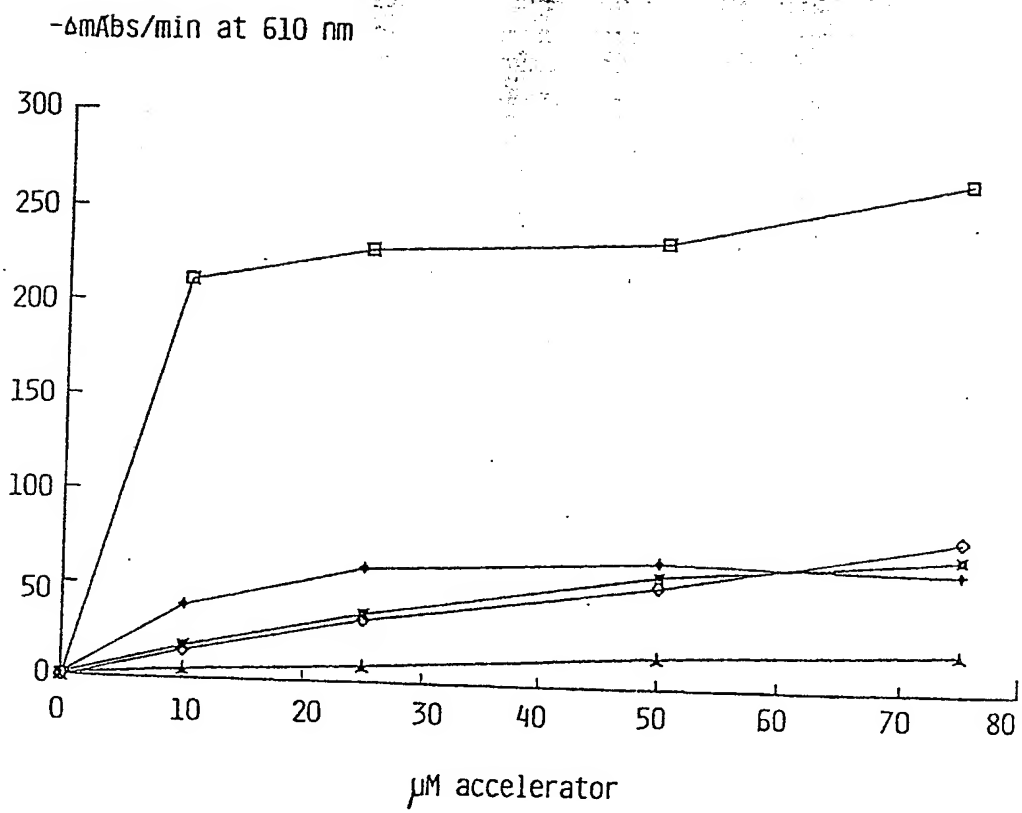
-ΔmAbs/min at 610 nm
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
80
μM accelerator

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00394

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12N 9/08, C11D 3/386, D06L 3/02
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N, C11D, D06L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, WPI, WPIL, CLAIMS, CHEMICAL ABSTRACT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DD, A, 147368 (PORSTMANN, BÄRBEL ET AL), 1 April 1981 (01.04.81), page 1 - page 2, line 3, claims	1-20
A	US, A, 3893803 (KAISER), 8 July 1975 (08.07.75), column 1, line 38 - column 2, line 7; column 4, line 12 - column 11, line 28	1-20
A	WO, A1, 9218687 (NOVO NORDISK A/S), 29 October 1992 (29.10.92)	1-20

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 1994	25 -03- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Anneli Jönsson Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00394

C (CONTINUATION) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A1, 9218683 (NOVO NORDISK A/S), 29 October 1992 (29.10.92) --	1-10

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

26/02/94

International application No.
PCT/DK 93/00394

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DD-A- 147368	01/04/81	NONE	
US-A- 3893803	08/07/75	JP-A- 49093156	05/09/74
WO-A1- 9218687	29/10/92	EP-A- 0580707	02/02/94
WO-A1- 9218683	29/10/92	NONE	